Analysis of dominant-negative mutations of the *Caenorhabditis elegans let-60 ras* gene

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The *let-60* gene of *Caenorhabditis elegans* controls the choice between vulval and hypodermal differentiation in response to an inductive signal from the gonad. *let-60* encodes a *ras* protein that acts downstream of the *let-23* receptor tyrosine kinase in a signal transduction pathway. Dominant-negative mutations of *let-60* [*let-60(dn)*] cause a reduction of the gene activity in *let-60(dn)*/+ heterozygotes and a vulva-less mutant phenotype. We have found that nine *let-60(dn)* mutations cause replacements of conserved residues. Four are in two novel positions; others are in positions known previously to cause dominant-negative mutations in mammalian cells. The locations of these lesions suggest that they disrupt the ability of the *ras* protein to bind guanine nucleotides. Four *let-60(dn)* mutant genes were introduced into wild-type animals in the form of extrachromosomal arrays and were found to generate three dominant phenotypes—lethality, vulva-less, or multivulva—depending on gene dose and alleles. The dominant lethality caused by high-dose transgenic *let-60(dn)* genes suggests a toxic effect of these mutant genes in early development. The dominant-negative effects of these mutations in heterozygotes are likely to be caused by competition between *let-60(dn)* and *let-60*(+) protein for a positive regulator. All *let-60(dn)* mutations interfere with *let-60(+)* activity, but some alleles have partial constitutive activity, suggesting that the ability to interact with the activator is separable from the ability to exert a physiological effect (stimulation of vulval differentiation). These *dn* mutations might be useful for interfering with *ras*-mediated signal transduction pathways in other multicellular organisms.

[*Key Words*: *ras*; *Caenorhabditis elegans*; dominant-negative mutations; vulval induction]

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Prior to vulval differentiation in *Caenorhabditis elegans* hermaphrodites, each of six vulval precursor cells (VPCs; posterior daughter cells of six ectodermal P cells, P3.p–P8.p) has the potential to generate either vulval cells or hypodermal cells. According to the current model, during vulval induction, three of the six precursor cells are triggered to generate vulval cells by an inductive signal from the anchor cell in the gonad (for review, see Horvitz and Sternberg 1991). In the absence of the inductive signal, the vulval cell fates for these precursor cells are repressed by an inhibitory signal from the large syncytial hypodermis (hyp7) mediated by the *lin-15* gene (Ferguson et al. 1987; Herman and Hedgecock 1990).

Genetic analysis indicated that *let-60* activity controls the choice of differentiation between the vulval and hypodermal cell fates in response to the intercellular signals (Fig. 1; Beitel et al. 1990; Han et al. 1990). Specifically, low *let-60* activity results in fewer than three of the six VPCs differentiating into vulval tissue (the vulva-less or Vul phenotype), while high *let-60* activity results in extra VPCs differentiating into vulval tissue (the multivulva or Muv phenotype). *let-60* encodes a *ras* protein with 83% of its first 164 amino acids (of a total 184) identical to those in the human N-*ras* protein (Han and Sternberg 1990). Genetic epistasis experiments suggest that *let-60 ras* acts downstream of *let-23*, a member of the epidermal growth factor (EGF) receptor family of tyrosine kinases (Aroian et al. 1990), in a signal transduction pathway (Han et al. 1990; Han and Sternberg 1990).

Gain-of-function mutations in *let-60 ras* [*let-60(gf)*] produce constitutively high *let-60* activity and a Muv phenotype (Ferguson and Horvitz 1985; Beitel et al. 1990; Han et al. 1990; G. Jongeward and P. Sternberg, unpubl.). The five independently isolated *let-60(gf)* mutations have the same DNA lesion at codon 13 (Beitel et al. 1990); such a change in mammalian *ras* leads to a decrease in the GTPase activity of the *ras* protein (for review, see Barbacid 1987). The gain-of-function (Muv) mutant phenotype can also be caused by extra copies of extrachromosomal wild-type *let-60 ras* gene (Han and

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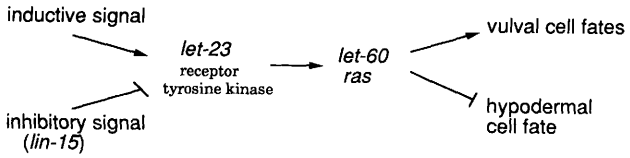

Figure 1. The role of *let-60 ras* in the control of vulval development. The level of *let-60 ras* activity controls the decision between vulval and hypodermal fates for each of the six vulval precursor cells in response to upstream intercellular signals (Beitel et al. 1990; Han et al. 1990). Specifically, low *let-60* activity results in hypodermal fate specification, whereas high *let-60* activity leads to vulval fate specifications. *let-60* activity, normally regulated by the upstream signals, is rendered constitutively low by *let-60 lf* or *dn* mutations, or constitutively high by *let-60 gf* mutations. *let-60 ras* has been proposed to act downstream of *let-23* (Han and Sternberg 1990; Han et al. 1990), which encodes a receptor tyrosine kinase (Aroian et al. 1990).

Sternberg 1990), suggesting that endogenous gene expression might be controlled quantitatively.

The opposite mutant phenotype, Vul, is caused by loss-of-function (*lf*) and dominant-negative (*dn*) mutations of *let-60 ras* (Beitel et al. 1990; Han et al. 1990). The nine *let-60(dn)* mutations cause a dominant Vul-less phenotype in hermaphrodites and a dominant defect in male tail morphology and mating ability. Eight of these mutations are recessive lethal at an early larval stage, consistent with the conclusion that *let-60* is also essential for larval growth based on analysis of *let-60(lf)* mutations. The dominant-negative effect in *let-60(dn)/+* heterozygotes is caused presumably by an interference of *let-60(+)* protein function by the *let-60(dn)* protein.

Previously, a number of mutations in guanine nucleotide-binding regions of *ras* proteins have been constructed or selected in vitro and found to have dominant-negative effects in mammalian or in yeast cells (Sigal et al. 1986; Feig and Cooper 1988; Powers et al. 1989). However, it is of interest to compare the in vivo-selected dominant-negative mutations of the *C. elegans let-60 ras* gene with those mutations studied previously.

In this study we determined the molecular lesions caused by the nine *let-60(dn)* mutations. We also determined the DNA alteration for two *let-60(dn)* intragenic revertants and found that one is an excellent candidate for a null mutation. Our results suggest that the *dn* mutations likely disrupt the ability of *let-60 ras* protein to bind guanine nucleotides and, therefore, prevent its activation. To understand the mechanism of the dominant-negative effect caused by these *let-60(dn)* alleles in heterozygotes, we further analyzed transgenic animals carrying exogenous *let-60(dn)* genes and examined the extent of vulval differentiation for two representative *let-60(dn)* mutations with various ratios of mutant to wild-type genes. We discuss the implications of these results as well as a model for competition between the *let-60(dn)* and *let-60(+)* proteins.

Results

All dominant-negative mutations of let-60 ras *likely cause defective guanine nucleotide binding*

We have determined the sequence alterations of all nine *let-60(dn)* mutations, after obtaining *let-60* DNA fragments from mutants by polymerase chain reaction (PCR) amplification (Table 1). For example, *let-60(sy101)* results in a substitution of arginine for glycine at position 10; we refer to this as *let-60(Arg-10)*. Figure 2 shows the three-dimensional crystal structure of the GTP-bound form of a truncated mammalian p21 *ras* protein (Pai et al. 1989, 1990; Tong et al. 1991). Because there are no gaps in the alignment between *let-60 ras* and mammalian *ras* proteins until after residue 179, the positions of *let-60(dn)* mutant residues likely correspond to those in

Table 1. *DNA and protein lesion of* let-60(dn) *and revertant mutations*

Class	Allele	Codon	nucleotide		amino acid	
			wild type	mutant	wild type	mutant
Dominant-negative (*dn*)[a]	*sy99*	10	GGA	AGA	Gly	Arg
	sy101	10	GGA	AGA	Gly	Arg
	n2301	15	GGT	AGT	Gly	Ser
	n1531	15	GGT	GAT	Gly	Asp
	sy94	16	AAA	AAT	Lys	Asn
	sy92	89	TCT	TTT	Ser	Phe
	sy95	89	TCT	TTT	Ser	Phe
	sy100	89	TCT	TTT	Ser	Phe
	sy93	119	GAT	AAT	Asp	Asn
Revertant of *dn*[b]	*sy163*	110	CCT	TCT	Pro	Ser
	sy127	123	CGA	TGA	Arg	Stop

[a]Genetic characterization of these *dn* mutations was described by Han et al. (1990) for alleles named *sy* and by Beitel et al. (1990) for alleles named *n*. *Phe-89 dn* is equivalent to the intragenic revertant (*n1981*) of an activated *let-60* mutant protein (*n1046* or *Glu-13 gf*). *Glu-13 Phe-89* protein does not act as an activated *ras* protein but has some dominant-negative activity (Beitel et al. 1990).
[b]These two alleles were isolated as intragenic revertants of *let-60(sy101 dn)* (Han et al. 1990) and appear to eliminate function of *let-60*.

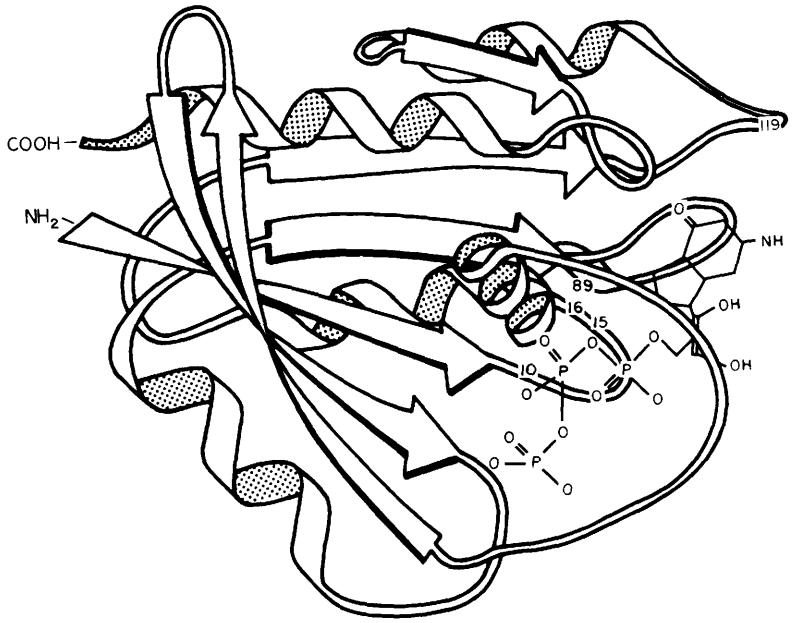

Figure 2. Three-dimensional structure of mammalian p21*ras*. The ribbon diagram of the structure of the GTP-bound form of a truncated p21*ras* (residues 1–166; Pai et al. 1989) is adapted from Bourne et al. (1991) and depicts the positions of amino acid residues that were altered by *let-60(dn)* mutations.

mammalian p21 *ras*. All of the *let-60(dn)* mutations alter amino acids that are invariant throughout evolution (Barbacid 1987; Han and Sternberg 1990) and appear to be in the region required for guanine nucleotide binding. Residues 10–17 form a loop in which main-chain amide hydrogens of several amino acids, plus the amino group of Lys-16, form hydrogen bonds with the α- and β-phosphates of GTP or GDP (Pai et al. 1990; Tong et al. 1991). Thus, Arg-10, Ser-15, Asp-15, and Asn-16 might disrupt the interaction of this loop with guanine nucleotides. Asp-119 is also required for GTP/GDP binding, as its carboxyl oxygens form hydrogen bonds with the guanine ring. Asn-119 is likely to disrupt these bonds. Ser-89 is not in direct contact with the guanine nucleotide, but its proximity to the binding site suggests that it might be important for stable binding. Phe-89 might disrupt such a role. Mutations in residues 16 and 119 of the mammalian *ras* protein cause a drastic (~100-fold) decrease in GTP/GDP-binding ability of the *ras* protein (Der et al. 1986; Sigal et al. 1986). We conclude that all of the *dn* mutations are likely to cause defective guanine nucleotide binding.

An intragenic let-60(dn) *revertant causes a premature termination codon and a null phenotype*

Previously, we have isolated two intragenic revertants of *let-60(sy101 dn)* (named *sy127* and *sy163*; Han et al. 1990). *let-60(sy101 sy127)* or *let-60(sy101 sy163)* double mutants are recessive lethal but have no dominant phenotypes: The *let-60(dn, revertant)*/+ heterozygotes have wild-type vulvae and normal male mating. The sequence changes of these two *let-60(dn)* intragenic revertants have been determined (Table 1). *sy163* converts residue 110 from the absolutely conserved proline to serine (*Ser-110*) while *sy127* produces presumably a carboxy-terminal-truncated protein by introducing a stop codon (TGA) at residue 123 (*Stop-123*). The sequence deleted in this truncated protein contains many conserved amino acids, including a region required for guanine nucleotide binding (Bourne et al. 1991), as well as the sites required for post-translational modifications and membrane attachment (Barbacid 1987; Gibbs 1991). Therefore, *Stop-123* is most likely a null mutation. *let-60(Arg-10 Stop-123)* homozygotes hatch but die during the early part of the first larval (L1) stage. Specifically, we observed eight animals under Nomarski optics and found that the gonad of an arrested animal consists only of the four primordial cells; the M mesoblast divided once at most, the P ectoblasts had not migrated to the ventral cord; and L1-specific alae are present in all arrested animals (Sulston and Horvitz 1977; Kimble and Hirsh 1979). On the basis of the phenotype under the dissecting microscope, *Stop-123* also produces the most severe recessive lethal phenotype among all of the loss-of-function mutations of *let-60 ras*; they have the smallest size on plates, and there are no "escapers" that survive to later stages, as is the case with other mutations (Clark et al. 1988; Beitel et al. 1990; Han et al. 1990). Therefore, animals with no zygotic *let-60* function arrest at the young L1 stage. It is possible that a maternal function of *let-60 ras* is required for animals to complete embryogenesis.

let-60(dn) *transgenic animals exhibit mutant phenotypes*

To analyze further the nature of these dominant-negative mutations, we reintroduced four different *let-60(dn)* genes into wild-type animals along with a dominant marker, resulting in the production of extrachromosomal DNA arrays (Table 2). The extrachromosomal arrays in transgenic animals are typically >1000 kb long.

Table 2. *Dominant-negative and* gf *phenotypes generated by extrachromosomal* let-60(dn) *DNA*

Allele	DNA[a] (μg/ml)	Number of animals injected	Number of transformants[b]		Phenotype[c]			Number of Ex genes[d]
			F_1	stable lines	% Vul	% Muv	*n*	
Arg-10	10	10	0	0				
Asn-16	10	10	6	0				
Phe-89	10	10	~50	7	80	0	92	140 ± 90
Asn-119	10	10	~100	0				
Arg-10	1	17	>400	>7	85	0	93	6 ± 1
Asn-16	1	10	>550	>10	63	19	178	7 ± 3
Asn-119	1	13	>580	>28	60	20	115	5 ± 1

[a]Only concentrations of injected *let-60* DNA are indicated. (The concentrations of marker and carrier DNA are described in Materials and methods.)

[b](F_1) F_1 progeny carrying injected DNA; (stable lines) germ-line transformants. The extrachromosomal DNA is maintained semi-stably in germ-line transgenic animals (Mello et al. 1991). A dominant lethal effect is indicated by lack of stable transformants in some injection experiments. Transformants can be routinely obtained by just injecting marker and carrier DNA. Previously, we have also shown that injection of a 5′- or 3′-truncated *let-60* gene does not cause any dominant effect. The dominant lethal phenotype is therefore unlikely to be caused by titration of transcription factors by multiple copies of its DNA-binding sites (Han and Sternberg 1990.)

[c]Phenotypes determined under a dissecting microscope for a representative transgenic line for each experiment. (% Vul) Percent vulvaless; (% Muv) percent multivulval; (*n*) number of animals counted.

[d]Approximate copy number (±1 S.D.) of extrachromosomal *let-60* genes in the transgenic lines estimated by PCR.

The structure of the arrays and the gene copy numbers in individual transforming lines remain relatively stable through numerous generations of growth (Mello et al. 1991). The ratio of DNAs in such extrachromosomal arrays is similar to the ratio of DNAs in the solution injected, so that the approximate number of copies of a test gene relative to marker and carrier DNA can be controlled (Mello et al. 1991).

When each of three *let-60(dn)* mutant genes (*Arg-10, Asn-16,* and *Asn-119*) were injected into wild-type animals at 1 μg/ml (~70 μg/ml of total DNA; see Materials and methods), viable transformants were obtained and the expected Vul phenotype was observed. However, if the concentrations were increased to 10 μg/ml, we were unable to obtain germ-line transformants from injection of any of the three mutant genes, indicating a dominant-lethal effect. In contrast, injection of a truncated *let-60* gene at 50 μg/ml does not cause lethality or any dominant phenotype (Han and Sternberg 1990). Therefore, it is possible that the *dn* mutations also affect dominantly *ras*-mediated pathways during early larval growth. The fact that a low dose of *let-60(dn)* causes a Vul, but not a lethal, phenotype suggests that *let-60 ras* function during vulval development is more sensitive to the change in its activity level than its essential functions during early development. At the same concentration (10 μg/ml), injection of *let-60(Phe-89 dn)* produces viable transformants containing extrachromosomal *let-60(Phe-89 dn)*, although a dominant Vul phenotype is associated with these transformants. This exception is probably the result of residual *ras* activity associated with the mutant protein, because the extrachromosomal *let-60(Phe-89 dn)* DNA can rescue the lethality of *let-60(Phe-89 dn)/ let-60(Phe-89 dn)* homozygotes. Partial vulval differentiation is also observed in *let-60(Phe-89 dn)* homozygotes carrying high copy numbers of the extrachromosomal *let-60(Phe-89)* gene. The residual activity of *let-60(Phe-89)* could be different qualitatively from the residual activity of *let-60(Asn-16)* or *let-60(Asn-119)*, because it does not cause a Muv phenotype, even when injected at high concentrations.

Surprisingly, when injected at 1 μg/ml, two *let-60(dn)* mutant genes also generated a dominant gain-of-function Muv phenotype in addition to the Vul phenotype (Table 2). Among a population of animals derived from the same transgenic line, some are multivulva, while others are vulvaless. The Muv phenotype of these transgenic animals might be caused by the residual activity of *let-60(Asn-119 dn)* and *let-60(Asn-16 dn)* proteins expressed from the extrachromosomal genes. Whether a transgenic animal displays the Vul or Muv phenotype might depend on relative level of the dominant-negative and residual activities. This hypothesis is supported by the fact that the *let-60(Asn-119 dn)* allele is recessive viable and allows partial vulval differentiation (see below and Table 3).

Arg-10 *and* Asn-119 *have distinct properties*

Because the transgene studies indicate two classes of dominant-negative mutations—those that cause a Muv phenotype (*Asn-16* and *Asn-119*) and those that do not cause a Muv phenotype (*Arg-10* and *Phe-89*)—we selected one of each class for further study. Vulval differentiation in strains containing the *Arg-10* and *Asn-119* mutations was analyzed further with Nomarski optics to observe the extent of vulval differentiation. As mentioned earlier, *let-60(Arg-10)* homozygotes are inviable

Table 3. *Dosage effect of two* let-60(dn) *alleles on vulval differentiation*

Genotype[a]	Vulval differentiation			
	% average (*n*)[b]	no. < 100%[c]	no. = 100%[c]	no. > 100%[c]
(*Arg-10,Stop-123*)/ (*Arg-10,Stop-123*)/+[d]	100 (13)	0	13	0
+/+/+	100 (20)	0	20	0
(*Arg-10*)/+/+	100 (20)	0	20	0
(*Arg-10*)/+	41 (22)	20	2	0
(*Arg-10*)/(*Arg-10*)/+	4 (17)	17	0	0
(*Arg-10*)/(*Arg-10*)	lethal			
(*Asn-119*)/+/+	33 (18)	15	3	0
(*Asn-119*)/+	2 (21)	21	0	0
(*Asn-119*)/(*Asn-119*)/+	79 (28)	10	15	3[e]
(*Asn-119*)/(*Asn-119*)	29 (28)	25	3	0
(*Asn-119*)/(*Stop-123*)	lethal			

[a]Only the *let-60* genotype is indicated. (The complete genotype of each strain is described in Materials and methods.) A duplication covering part of chromosome IV (*nDp5*; Beitel et al. 1990) was used for the additional wild-type copy of *let-60*.
[b]Percentage of vulval precursor cells generating vulval cells relative to wild type (100%), examined under Nomarski optics (defined by Han et al. 1990; see Materials and methods). The numbers of animals examined are indicated (*n*). Two strains listed show an early larval lethal phenotype; their vulval cell differentiation cannot be examined. Animals of genotype *let-60*(*Arg-10 Stop-123*)/+ or *Df*/+ have been shown previously to have wild-type vulval development (Han et al. 1990; Beitel et al. 1990). In strains heterozygous for *let-60*(*Asn-119 dn*) and *let-60*(+), both mutant and wild-type genes contribute to the total activity, although *let-60*(+) activity is greatly reduced by the strong dominant negative effect of *let-60*(*Asn-119 dn*). It is also possible that stability of the mutant proteins is different from that of the wild-type protein such that the quantity of the mutant proteins are also different from wild type.
[c]Number of examined animals having <100% vulval differentiation, 100% vulval differentiation, or >100% vulval differentiation, respectively. Animals having 100% vulval differentiation do not necessarily have wild-type vulva.
[d]*Stop-123* is a putative null mutation (see text).
[e]Three of these 28 *let-60*(*Asn-119*)/*let-60*(*Asn-119*)/+ animals showed >100% vulval cell differentiation (more than three vulval precursor cells generate vulval cells). In these animals, in addition to P5.p, P6.p, and P7.p, P4.p (one animal) or half of the P*n*.p progeny (P4.pa, two animals) differentiated into vulval cells.

while *let-60(Asn-119)* homozygotes are viable. A chromosomal duplication (*nDp5*; Beitel et al. 1990) was used to alter the ratio between mutant and wild-type gene dose (Table 3). We found that changes in the endogenous gene ratio between *let-60*(+) and *let-60(Arg-10 dn)* genes cause drastic changes in the extent of vulval differentiation. In particular, an additional wild-type gene in *dn*/+/+ animals suppresses the *dn* toxic effect of *let-60(Arg-10 dn)* completely in *dn*/+ animals (from 41% vulval differentiation to 100%). An additional mutant gene reduces the average differentiation to 4% in *dn*/*dn*/+ animals (Table 3). Therefore, *let-60(Arg-10 dn)* contains no or little activity by itself but interferes with the function of the wild-type gene product.

The *Asn-119 dn* mutation has more complex effects on *let-60 ras* activity (Table 3). First, *let-60(Asn-119 dn)* exerts a stronger dominant-negative effect than *let-60(Arg-10 dn)*: Average vulval differentiation for *Asn-119*/+ and *Asn-119*/+/+ animals is 2% and 33%, respectively. Second, *let-60(Asn-119 dn)* has residual activity: *let-60(Asn-119 dn)* homozygous animals are recessive viable and have an average of 29% vulval differentiation. Third, the lethal phenotype of *let-60(Asn-119 dn)* in *trans* to the presumptive null mutation [*let-60(Arg-10 Stop-123)*; see above] suggests that the residual activity is dose dependent. Two copies of *let-60(Asn-119 dn)* are sufficient for viability, but one copy is not. Also, one additional copy of *let-60(Asn-119 dn)* increases vulval differentiation: *let-60(Asn-119 dn)/let-60(Asn-119 dn)* homozygous animals have more vulval differentiation (29%) than do *let-60(Asn-119 dn)*/+ heterozygotes (2%), and *dn/dn*/+ animals have more vulval differentiation (79%) than do *dn*/+/+ animals (33%). Finally, some multivulva animals (i.e., >100% vulval differentiation) were observed among the *let-60(Asn-119)/let-60(Asn-119)*/+ animals. We found that vulval differentiation in these animals is partially signal independent. The gonads of nine animals of this genotype were ablated at the L1 stage with a laser microbeam to eliminate the source of inductive signal. Three of the animals had a total of five and one-half VPCs (for definition, see Materials and methods) differentiating into vulval tissue, suggesting that *let-60* activity in the strain is partially constitutive. Therefore, the *let-60(Asn-119 dn)* gene product has a very strong dominant-negative effect on wild-type activity but has some constitutive activity by itself to trigger vulval differentiation.

The dominant-lethal effect of high-dose transgenic let-60(Arg-10 dn) *is suppressed by the presence of the genomic* let-60(Glu-13 gf) *gene*

The dominant-negative effects of *dn* alleles are suppressed completely by *gf* alleles in *let-60(dn)/let-60(gf)*

animals (Beitel et al. 1990; Han et al. 1990), indicating that *dn* protein cannot compete with *gf* protein when there is one copy of the *dn* and one copy of the *gf* mutant genes. Because we can drastically alter the relative dose, transgenic animals carrying exogenous *let-60(Arg-10 dn)* allow a more stringent test of the hypothesis that *dn* protein cannot interfere with activated *ras*.

The DNA array carrying high copy numbers of *let-60(Arg-10 dn)* genes was generated in a *let-60(gf)* background, and the segregants of hermaphrodites of genotype *let-60(gf)/+*; *Array* were examined for the production (and proportion) of lethal and nonlethal transgenic progeny. Table 4 lists the genotypes and phenotypes of transgenic animals segregating from a single *let-60(Glu-13 gf)/+* heterozygote carrying a *let-60(Arg-10 dn)* extrachromosomal array injected at 10 μg/ml (see Table 2). As described above, the injected *let-60(Arg-10 dn)* genes cause a dominant-lethal phenotype in a wild-type background: None of the 22 transgenic progeny from a *gf/+* heterozygote has a +/+ genotype (one-fourth is expected without the *dn* effect). However, this lethal phenotype can be weakly suppressed by one copy of *let-60(Glu-13 gf)* in *gf/+* heterozygotes and strongly suppressed by two copies *let-60(Glu-13 gf)* in *gf/gf* homozygotes. Among the transgenic progeny of *gf/+* animals carrying extrachromosomal *let-60(Arg-10 dn)* genes, we observed twice as many *gf/gf* homozygotes (15) as *gf/+* heterozygotes (7) (Table 4), whereas normally we would only expect a ratio of 1 *gf/gf* : 2 *gf/+* : 1 +/+. The Vul phenotype caused by *let-60(Arg-10 dn)* is also suppressed completely. Suppression of the *dn* mutant phenotype by *gf* mutations has been reported previously in yeast cells with either yeast or mammalian *ras* (*dn*) genes (Sigal et al. 1986; Feig and Cooper 1988; Powers et al. 1989). The constitutive activity of activated *ras (gf)* mutant proteins is most likely the result of a decrease in GTPase activity (Barbacid 1987). Therefore, failure of *let-60(dn)* protein to compete with *let-60 (gf)* protein suggests that the dominant-negative effect of the *dn* mutant proteins on wild-type activity is probably the result of disruption of the activation of wild-type *ras*, rather than disruption of its interaction with an effector protein.

Table 4. let-60(Glu-13gf) *suppresses the dominant-negative effect of extrachromosomal* let-60(Arg-10 dn)

F_1 progeny[a]			Average F_2 progeny	
genotype	no. of animals	F_1 phenotype[b]	nontransgenic	transgenic
gf/gf	15	Muv or WT	92	23
gf/+	7	Muv or WT	34	4
+/+	0			

[a]Transgenic progeny of a single *let-60(Glu-13 gf)/dpy-20* animal carrying extrachromosomal copies of the *let-60(Arg-10 dn)* gene were picked at the L4 larval stage and their genotypes were determined by examining their F_2 progeny. *let-60(Arg-10 dn)* DNA was injected at 10 μg/ml along with marker DNA. The non-Mendelian segregation pattern of the genotype indicates a lethal effect caused by extrachromosomal *let-60(Arg-10 dn)* and a suppression effect by *let-60(Glu-13 gf)*.

[b]Vulval phenotype under dissecting microscope. (Muv) Multivulva; (WT) wild type. Nine of 15 F_1 *gf/gf* transgenic animals were Muv; two of seven F_1 *gf/+* transgenic animals were Muv. There were no Vul phenotypes observed among all transgenic animals.

[c]Average number of F_2 progeny of individual F_1 transgenic animals. The transgenic animals show the marker phenotype (Roller). The relatively large numbers of progeny associated with *gf/gf* animals indicate the stronger suppression of the lethality by a high-copy number of *let-60(Arg-10 dn)* genes. The number of nontransgenic F_2 progeny reflects the fertility of F_1 animals. The genotypes of F_2 transgenic progeny of F_1 *gf/+* transgenic animals were not determined (they are expected to be a mixture of *gf/gf* and *gf/+* in a ratio similar to that of F_1 progeny). No vulvaless animals were detected among any of the F_2 progeny; 166 of 232 progeny of *gf/gf* F_1 transgenic animals were Muv.

Discussion

We have demonstrated that nine *dn* mutations in *C. elegans let-60 ras* gene [*let-60(dn)*] cause changes in five conserved amino acids that are required for guanine nucleotide binding in mammalian *ras* proteins. Two of these positions are new. Because *gf* (or "activated") and some of the *dn* mutations of *let-60 ras* correspond to positions that result in *gf* and *dn* mutations in mammalian and yeast *ras*, it is likely that *let-60 ras* has many of the biochemical functions of these other *ras* proteins (e.g., GTP/GDP binding and GTP hydrolysis). Disruption of these functions leads to specific developmental defects in *C. elegans*. We have also demonstrated that these *let-60(dn)* mutant genes can cause various dominant mutant phenotypes in transgenic animals. These phenotypes suggest that different properties are associated with some of these *dn* mutations even though each mutation interferes with *let-60(+)* function in vulval development. Our further dose analysis shows that the dominant-negative effect of a mutant gene is separable from its ability to be activated, because *let-60(Asn-119)* has the strongest toxic effect on *let-60(+)* but, nonetheless, has partial constitutive activity to induce vulval differentiation as well as support larval growth.

Disruption of the essential roles of let-60 ras *by* dn *mutations*

Because the putative *let-60* null mutation causes animals to arrest during the early part of the first larval stage, *let-60* is required for postembryonic growth and development. Similar larval lethality is seen for most *let-60(dn)* homozygotes, suggesting that they are impaired severely in the essential function of this gene. However, in *let-60(dn)/+* heterozygotes, *let-60* function during vulval development, but not early larval growth, is disrupted: Viable *dn/+* animals often have no vulval differentiation (Beitel et al. 1990; Han et al. 1990). Therefore, it is possible that the dominant-negative effect is tissue specific. The results described here suggest that such an explanation is unlikely because dominant le-

thality can also be produced in wild-type animals by the presence of a high copy number of *let-60(dn)* genes in transgenic animals. At least for *let-60(Arg-10 dn)*, this lethality is likely caused by a strong dominant-negative effect that leads to a decrease in *let-60(+)* activity. Therefore, the selective *dn* effects (disrupting vulval development but not growth) in *let-60(dn)/+* animals are the result of differences of sensitivity to the decrease in *let-60 ras* activity between these two aspects of development. A similar observation has been made for the gain-of-activity mutant phenotype caused by extra copies of *let-60(+)* genes in transgenic animals (Han and Sternberg 1990). Early larval growth thus appears to be less sensitive to the changes in *let-60* activity than is vulval development.

Mechanism of the dominant-negative effect of let-60(dn) *mutations*

The amino acid changes caused by *let-60(dn)* mutations likely disrupt the normal interaction between *let-60 ras* protein and GDP or GTP, resulting in loss of protein function. Such a defect easily explains the recessive-lethal phenotype. How do these mutant *ras* proteins, with presumably a decreased ability to bind to guanine nucleotides, inactivate the wild-type *let-60 ras* protein in a *let-60(dn)/+* heterozygote? Two common models can be used to explain a dominant-negative mutant effect (Herskowitz 1987): Either the mutant protein exerts a toxic effect in a heteromultimer or the mutant protein competes with the wild-type protein for another factor. According to the first model, *let-60 ras* protein would form multimers through direct or indirect interactions. For example, multiple *ras* protein molecules may form a functional protein complex by interacting with another factor. In this model the dominant toxic effect of a *dn* mutant protein is exerted by inactivating a complex containing both the wild-type and *dn ras* proteins. If such a complex contained more than two *let-60* molecules, this model would be consistent with all of our molecular genetic data on *let-60(dn)*, including the Vul phenotype of *let-60(Asn-119 dn)/+/+* animals (Table 3). However, there is no compelling evidence that *ras* proteins form multimeric complexes.

The second model proposes a competition between the wild-type and mutant protein for another positive factor (Fig. 3). This competition results in reduction of the *let-60(+)* activity in *let-60(dn)/+* heterozygous animals. To explain the severe Vul phenotype generated by *let-60(dn)* mutations in *dn/+* heterozygotes [especially in the case of *let-60(Asn-119)*, in which vulval differentiation in *Asn-119/+/+* animals is only 33%], two assumptions need to be made. First, the cellular concentration of this positive factor must be limiting and *let-60 ras* must be in excess in cells responding to the inductive signal (for further discussion, see below), so that *let-60(dn)* protein could titrate the factor. Second, *let-60(dn)* proteins must form a more stable complex with the factor than the wild-type *let-60 ras* protein. This positive factor could either be an upstream activator or a downstream target protein. Because the effect of a dominant-negative mutant can be suppressed by the presence of constitutively active *ras* protein [*let-60(Glu-13 gf)*], the *let-60(dn)* and *let-60(+)* proteins likely compete for an activator rather than a target protein. *ras* protein is activated through a change from the GDP-bound state to the GTP-bound state and is deactivated by GTP hydrolysis (Fig. 2; Bourne et al. 1991). Therefore, this activator could be a guanine nucleotide exchange factor (GNEF; Downward et al. 1990; Jones et al. 1991; Kaibuchi et al. 1991). We speculate that binding of *ras(dn)* mutant protein (presumably the guanine nucleotide-free protein) to GNEF prevents the release of the GNEF and depletes the pool

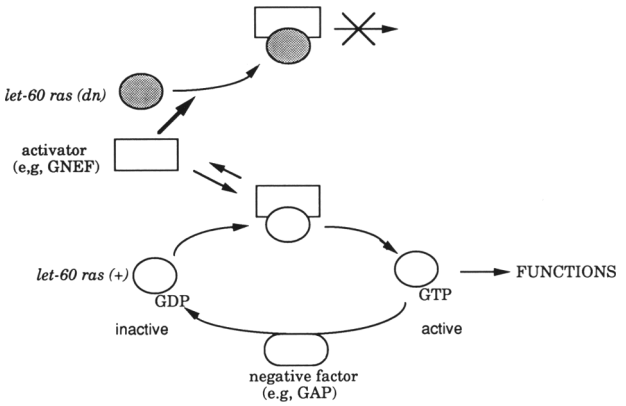

Figure 3. A model for the dominant-negative effect of *let-60(dn)* mutant proteins. The mutant protein competes with the wild-type protein for an interacting factor, likely an activator. The amount of this activator is proposed to be limiting relative to the amount of *let-60 ras* protein, and this activator forms more stable complexes with *let-60(dn)* protein than with *let-60(+)* protein. Thus, the activator can be titrated by *let-60(dn)* protein molecules that are inactive as a result of the loss of their ability to bind guanine nucleotides. The Vul phenotype in *let-60(dn)/let-60(+)* heterozygotes is caused by such a reduction in *let-60(+)* activity. Because *ras* is activated by changing from a GDP-bound state to a GTP-bound state, this activator could be a GNEF (for review, see Bourne et al. 1991). The studies with *S. cerevisiae RAS* and *CDC25* proteins (function as a GNEF) provide evidence for such a proposal (Powers et al. 1989; Créchet et al. 1990; Jones et al. 1991). Although we propose that the *let-60 ras* is in excess relative to the upstream activator, its amount may be normally limiting relative to a negative regulator. An increased dose of *let-60(+)* in transgenic animals causes hyperactivity and a Muv phenotype. *ras*-GTP is deactivated by GTP hydrolysis and requires the function of GAP (McCormick 1989; Bourne et al. 1991). Such a GAP protein may exist in *C. elegans* to normally limit the activity of *let-60 ras*. An increase in the dose of the wild-type *ras* gene might overwhelm the negative activity of GAP, causing the *gf* phenotypes (Han and Sternberg 1990). In wild type, the balance between *ras* activation and deactivation may be controlled by an upstream inductive signal through the *let-23* receptor tyrosine kinase. The activated *let-23* kinase may either stimulate the function of a GNEF or downregulate the activity of a GAP protein. Either effect would lead to activation of *let-60 ras*. *let-60(dn)* mutations decrease the rate of *ras* activation constitutively and produce a *lf* phenotype regardless of the presence of the upstream signal.

available to interact with, and hence activate, wild-type *ras* protein.

A competition model has been used previously to explain the dominant-negative effect of *ras* mutations in mammalian cells and yeast (e.g., *Asn-16, Ala-17, Asn-119* of mammalian *ras* p21 protein and *Pro-22* and *Ala-25* of yeast *RAS2* protein) (Sigal et al. 1986; Feig and Cooper 1988; Powers et al. 1989). Particularly, the work done by Powers et al. (1989) suggested that *CDC25* of *Saccharomyces cerevisiae*, a yeast GNEF (Créchet et al. 1990; Jones et al. 1991), is the limiting factor and is titrated by binding to *ras*(+) or *ras(dn)* proteins. Increased expression of the *CDC25* gene in yeast can suppress the toxic effect caused by a dominant-negative mutation in either the yeast *RAS2* gene (Ala-22) or the mammalian H-*ras* gene (Ala-15) in the presence of wild-type yeast *ras* protein. Furthermore, because increased dosage of the wild-type *RAS2* gene neither causes a dominant phenotype nor obviously suppresses the dominant-negative effect of one *dn* mutation in yeast, there is no evidence that *ras* is a limiting factor in yeast.

Although the competition model is consistent with our molecular and genetic data on *let-60(dn)* mutants, so far there is no direct evidence for it in either *C. elegans* or mammalian cells. Also, a downstream effector protein is not excluded from being the limiting factor that is titrated out by some, if not all, of the dominant-negative *let-60 ras* mutant proteins: Suppression of *let-60(dn)* by *let-60(Glu-13 gf)* may be the result of an even higher affinity of *let-60(Glu-13 gf)* for the effector. In yeast, suppression of the *dn* phenotype by increasing the level of *CDC25* might simply be the result of increase in the amount of *ras*–GTP, which can compete with *ras(dn)* for the effector.

Negative regulation of let-60 ras

Our molecular genetic analyses of the *let-60* gene suggest that its product is a limiting factor in signal transduction. The extent of vulval differentiation is sensitive to the number of copies of wild-type or mutant *let-60* genes (Table 3; Beitel et al. 1990; Han and Sternberg 1990). In mammalian cells, an increased dose of wild-type *ras* genes causes malignant transformation (Chang et al. 1982; Pulciani et al. 1985; McKay et al. 1986; Quaife et al. 1987). The dose effect of the *let-60 ras* protein may be the result of its interaction with a negative regulator. For example, *ras* activity is known to be negatively regulated by GAP (GTPase-activating protein), which catalyzes the GTP hydrolysis of the *ras*–GTP complex (for review, see McCormick 1989; Bourne et al. 1991). The cellular *ras* concentration might be limited relative to the GAP activity. An increase in dose of the wild-type *ras* gene might overwhelm this negative activity of GAP or decrease the rate of GAP-stimulated GTP hydrolysis and thus cause a *gf* mutant phenotype. This hypothesis is consistent with the observation that overexpression of GAP can suppress *c-ras*-mediated transformation in mammalian cells (Zhang et al. 1990).

Mechanism of let-60(Asn-119 dn) *functions*

The *let-60(Asn-119 dn)* gene product has the strongest dominant-negative effect on *let-60*(+) activity but contains some constitutive activity itself to trigger vulval differentiation and to support larval growth (Table 3). The residual activity of *let-60(Asn-119 dn)* protein in the vulval signaling pathway is consistent with the properties of mammalian *ras* proteins with changes in residue 119. For example, *ras* proteins with residue 119 changed from Asp to Ala or His can cause malignant transformation in mammalian cells, although these mutant proteins cause a drastic decrease in affinity for GDP/GTP (Der et al. 1986; Sigal et al. 1986). The constitutive nature of the residual activity associated with changes in residue 119 may be due to a decrease in affinity of *ras* for guanine nucleotides that leads to an increase in the exchange rate from *ras*–GDP to *ras*–GTP (i.e., causes GNEF-independent *ras* activity; Sigal et al. 1986). Therefore, the complicated phenotypes of *let-60(Asn-119 dn)* may be the result of two separable and abnormal biochemical functions of the protein: On one hand, *let-60(Asn-119)* protein (possibly free of guanine nucleotides) might form a very stable complex with an activator (e.g., GNEF), preventing it from interacting with the *let-60*(+) protein (see Fig. 3); on the other hand, *let-60(Asn-119)* has its own low activity that does not require the activation by GNEF and is less sensitive to the deactivation by GAP.

Regulation of let-60 *by the inductive signal*

During vulval induction, how is *let-60 ras* activity regulated by the upstream inductive signal and the receptor tyrosine kinase encoded by the *let-23* gene (Aroian et al. 1990)? The upstream signal may either activate a GNEF that promotes the exchange of *ras* from GDP- to GTP-bound form or down-regulate the activity of GAP, which promotes GTP hydrolysis by *ras*. In the absence of upstream kinase activity, the activation step would be limiting, but a high dose of wild-type *ras* might reverse the situation. In the presence of the signal, the deactivation step would be limiting, but dominant-negative *ras* mutations might reverse the situation. This model predicts that overexpression of GNEF in wild-type *C. elegans* will cause a multivulva phenotype.

It is also possible that the in vivo regulation of *ras* activity in these *let-60(dn)* heterozygotes is more complicated, perhaps involving the combination of a number of positive and negative-regulatory factors or a protein complex that exists in a number of distinct states (see legend to Fig. 3). Understanding how these dominant-negative mutations act should provide insights into the mechanism by which *ras* proteins exert their signal-transducing functions.

Utility of dominant-negative mutations

Dominant-negative mutations can be a useful tool for study in vivo of functions of cloned genes (Herskowitz

1987). For example, *dn* mutations of the *Xenopus* fibroblast growth factor (FGF) receptor have suggested a role of this protein in the patterning of the early *Xenopus* embryo (Amaya et al. 1991). We have shown that particular *ras dn* mutations (e.g., *Arg-10*) disrupt the function of *ras* during vulval development in transgenic animals. Such simple *dn* mutations might be useful to elucidate the function of *ras* in the development of other organisms.

Materials and methods

General methods

Methods for culturing, handling, and genetic manipulation of *C. elegans* were as described by Brenner (1974). All genetic experiments were performed at 20°C. Methods for analysis of vulval defects under dissecting microscope and under Nomarski optics were as described previously (Han and Sternberg 1990; Sulston and Horvitz 1977). The genetic nomenclature used was as described (Horvitz et al. 1979). *let-60(dn)* strains were described by Han et al. (1990) and Beitel et al. (1990). Other strains were constructed according to standard methods.

Determining the nucleotide changes in let-60 *mutants*

DNA fragments containing *let-60*-coding regions were obtained by polymerase chain reaction (PCR) amplification from homozygous mutant animals (which are dead larvae, in most cases). The method for PCR amplification of DNA from dead larvae was as described by Beitel et al. (1990). Sequences of the mutant DNA were determined by one or both of the two methods: In most cases, PCR-amplified DNA fragments were directly sequenced after gel purification (Kretz et al. 1989); in some cases (exon 1 of *sy94* and *sy101*; exons 2 and 3 of *sy92*, *sy93*, *sy95*, and *sy100*), PCR fragments were subcloned (see below) and then sequenced. In later cases, the DNA lesion of each mutation was confirmed by sequencing multiple clones, or in the case of *sy94* and *sy101*, by also sequencing gel-purified amplified fragments. All four exons have sequenced for all 11 alleles listed in Table 1. Four determined DNA lesions (representing seven genetically isolated *dn* alleles) were also tested functionally for their *dn* effects by microinjection experiments (Table 2). There had been ambiguity of the *dn* allele (either *sy101* or *sy94*) used to generate *sy127* (Han and Sternberg 1990). Our sequence analysis showed it to be *sy101*, and we will thus refer it as *sy101 sy127* (or *Arg-10 Stop-123*).

In vitro construction of let-60(dn) *genes*

New plasmids were constructed to facilitate the construction of mutant *let-60* genes. pMH105 was first generated by deleting part of the linker region (between *Eco*RI and *Apa*I) of vector Bluescript (SK+). This deletion was achieved by digesting the Bluescript (SK+) with *Eco*RI and *Apa*I and then self-ligating the large fragment after end-filling with T4 polymerase. A 6.8-kb *Bam*HI–*Xho*II genomic fragment containing the entire *let-60* gene (Han et al. 1990) was inserted into the *Bam*HI site of pMH105. The resulting plasmid, called pMH106, was used subsequently for subcloning *let-60(dn)* fragments. For alleles located in exon 1, the *Hin*dIII–*Apa*I fragment of pMH106 was replaced by mutant DNA fragments; for alleles located in exon 2 or 3, the *Apa*I–*Eco*RV fragment of pMH106 was replaced by mutant DNA fragments.

Microinjection transformation

Each construct was injected into *dpy-20(e1282)* (Hosono et al. 1982) hermaphrodites together with pMH86 (containing the *dpy-20* gene) (15–25 μg/ml). To keep total DNA concentration approximately the same, Bluescript (SK+) plasmid was coinjected at 50 μg/ml. Because of the large number of F_1 transformants (non-Dpy animals) produced, the number of F_1 transgenic animals listed in Table 2 is approximate. For some experiments described in Table 2, about four to eight F_1 transformants were pooled on a single plate for screening for stable lines. Each plate containing F_2 transformants was scored as one stable line; thus, the number of stable lines listed in Table 2 is a minimum.

For the experiments described in Table 4, a dominant *rol-6* mutant gene (pRF4; Mello et al. 1991; 50 μg/ml) and *dpy-20* gene (pMH86; 10 μg/ml) were coinjected into *let-60(n1046 gf)/dpy-20(e1282)* hermaphrodites along with 10 μg/ml of *let-60(sy101 dn)* DNA (pMH136). pMH86 is a Bluescript (SK+)-derived plasmid that has a 6-kb *Xba*I DNA insert containing the entire *dpy-20* gene (subcloned from a *dpy-20*-containing cosmid; D. Clark and D. Baillie, pers. comm.). Because the Dpy phenotype of *dpy-20* homozygotes, which normally suppresses the roller (Rol) phenotype, is rescued in the transgenic animals by the extrachromosomal *dpy-20* gene, the dominant Rol phenotype is associated with all transgenic animals.

Estimation of copy numbers of extrachromosomal let-60 *genes*

To estimate extrachromosomal copy number, a *let-60* DNA fragment was amplified by PCR from L4-stage stable transgenic animals as well as nontransgenic *dpy-20* animals. To keep PCR amplification in a linear range, only 15 rounds of amplification were performed (Robinson and Simon 1991). The PCR products were loaded on an agarose gel, and the amount of amplified DNA was detected by Southern analysis. As a control, primers were also added to each reaction to amplify a DNA fragment located in the *let-23* gene (chromosome II) (Aroian et al. 1990). For each transgenic line, three to four animals were first lysed with proteinase K in 20 μl. The lysate (2× 2 μl and 2× 5 μl) was then added to one of the four tubes containing the PCR mix. The relative intensity of the two DNA bands on an autoradiograph was measured by a densitometer (LKB). The number of extrachromosomal *let-60* genes listed in Table 2 is the average of the four measurements for each transgenic line.

Strain construction

To construct strains containing *nDp5* and *let-60(sy101 dn)*, *dpy-20(e1362) unc-22(e66)/dpy-20(e1362) unc-22(e66); nDp5; him-5(e1490)*, males (Beitel et al. 1990) were crossed with *let-60(sy101 dn) dpy-20(e1282)/let-60(n1046 gf) unc-22(s7)* hermaphrodites. Non-Dpy non-Unc F_1 cross progeny were picked and placed individually on new plates. F_1 progeny of the genotype *let-60(sy101 dn) dpy-20(e1282)/dpy-20(e1362) unc-22(e66); nDp5* were selected by analyzing F_2 segregants. A strain of genotype *let-60(Arg-10 dn) dpy-20(e1282)/let-60(sy101 dn) dpy-20(e1282); nDp5* was obtained from screening the F_2 progeny. The complete genotype for *let-60(sy101 dn)/+* animals is *let-60(sy101 dn) dpy-20(e1282)/let-65(s254) unc-22(s7) unc-31(e159)* (Han et al. 1990). To construct strains containing *let-60(sy93 dn)* and *nDp5*, a strain of genotype *unc-24(e138) mec-3(e1338) dpy-20(e1282)/unc-24(e138) mec-3(e1338) dpy-20(e1282); nDp5; him-5(e1490)* was constructed first. Males of this strain were then crossed with *unc-24(e138) let-60(sy93dn)/unc-24(e138) let-60(sy93 dn)* hermaphrodites (Han et al. 1990).

The F_1 non-Unc cross progeny were picked and have the genotype *unc-24 let-60(sy93 dn)/unc-24 mec-3 dpy-20(e1282); nDp5; him-5(e1490)/+*. A strain of genotype *let-60(Asy93 dn)/unc-24 let-60(sy93 dn); nDp5* was obtained from F_2 or F_3 progeny.

Examining vulval differentiation with Nomarski optics

For *let-60(dn)/+/+* and *+/+/+* animals, self progeny (late L3 or early L4 larvae) of *let-60(dn)/+/+* hermaphrodites were first examined for vulval induction, and their genotypes were determined by progeny testing. Except for *+/+/+* animals, vulval differentiation was determined by examining the progeny from mothers of the same genotype.

The percentage of vulval differentiation is determined as the percentage of vulval precursor cells (P3.p–P8.p) differentiating into vulval cell type relative to wild type (as defined by Han et al. 1990). In a completely vulvaless animal, each of the six precursor cells divides once, and their progeny appears to fuse with the syncytial hypodermis. The vulval differentiation in such an animal is said to be 0%. In a wild-type hermaphrodite (100% vulval differentiation), three of the six precursor cells divide further than the first round of division, producing the progeny characteristic of vulval cell types (Sulston and Horvitz 1977; Sternberg and Horvitz 1986). Animals with fewer than three cells differentiating to vulval cell types have <100% vulval differentiation (vulvaless); animals with more than three precursor cells differentiating to vulval cell types have >100% vulval differentiation (multivulva). Sometimes only one of the two daughters of a precursor cell divided further to generate vulval tissue; the vulval differentiation in this case is "one-half cell". Laser ablation experiments were performed as described (Sulston and White 1980).

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